On the Relationship between Hæmolysis and the Phagocytosis of Red Blood Cells.

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The nature of the substance or property in normal as well as in immune serum which induces phagocytosis has been of late a matter of considerable discussion, and the chief point of controversy has been whether phagocytosis is caused by some well-known immune substance, or whether it is brought about by something which until recently had not been completely recognised as a product of immunisation processes, *e.g.*, the "opsonin" of Wright and Douglas.

Whatever the nature of this substance may be, it seems established beyond doubt that it acts on the bodies phagocytosed, the stimulin theory of Metchnikoff and his school having given way to the theory supported especially by Wright and other observers in this country, that the action is on the bodies phagocytosed and not on the phagocytes, notwithstanding the work of Löhlein (1), Leishman (2) and Besredka (3).

Wright and Douglas (4 and 5) in their well-known work on this subject, described this property of the serum as being due to a body which up to that time had not been properly recognised. To this they gave the name "opsonin," and by their ingenious experiments they rendered clear and concrete what had been before but nebulous and ill-defined.

They as well as Bulloch and Atkin (6) and Hektoen and Ruediger (7) described this body as being thermolabile from the fact that it was to a large extent destroyed by heating the serum to 55° C. to 65° C. Dean (8) repeated this work, using a somewhat different technique, and having found that in normal, but especially in immune sera, a certain amount was not destroyed, decided to call it thermostable. As Wright (9) has since pointed out, this is merely a matter of terms; but from his as well as from Dean's experiments it is clear that a very large amount of destruction takes place at these temperatures.

Dean at the same time put forward the view, shared chiefly by workers in the Pasteur Institute in Paris, that the substance or property in the serum described by Wright and Douglas was not new but had been well known before, and Dean laid stress on the work of Savtchenko (10) on the phagocytosis of red blood cells, pointing out that this property had been attributed by Savtchenko to the "fixateur."

As there seems to have crept into this question some doubt as to the

exact interpretation to be put on Savtchenko's work, and particularly as to the exact significance of the term "fixateur" as used by him, it is necessary to briefly consider his position, especially as Barratt (11) has put a different interpretation on it from Dean.

Savtchenko assumed that the laws regulating the action of cytotoxins were entirely analogous to those regulating the action of immunising substances on microbes, and considered that experiments on phagocytosis might be permissibly conducted with animal cells and adopted red blood cells as being easy to work with.

This opinion would indicate that Savtchenko considered that the action of hæmolysis was the analogue of that of immunising substances on microbes, since a cytotonic action with reference to red blood cells would mean hæmolysis. This is also indicated further on in his work when he says that as has been pointed out by Bordet, when the red blood cells of an animal A are injected into an animal B, the serum of the latter becomes toxic for the red blood cells of the former, and that he himself has established a complete analogy between the action of the serum on the red blood cells and that of the immune specific serum on the microbe as well in the animal body as "in vitro."

Further he says,* "Dans le sérum spécifique se trouve une substance ou fixateur (d'après la terminologie de Metchnikoff) qui se fixe sur les globules rouges correspondants—ou bien sur les microbes—et par son action prépare ces derniers à leur dissolution par les alexines (cytases) qu'on trouve dans chaque sérum. Le fixateur ne se détruit pas à 55° C. à 60° C. Ehrlich et Morgenroth ont montré que le fixateur a une affinité spécifique pour les globules rouges correspondants, et qu'une fois fixé sur eux, il ne s'en détache pas dans les lavages ultérieurs, ainsi que dans la centrifugation dans l'eau physiologique. Si l'on soumet les globules rouges ainsi traités à l'action du sérum normal contenant des alexines, ils se dissolvent."

With regard to Metchnikoff's definition of the fixateur which Savtchenko accepts, one may state what Metchnikoff (12) himself has given in his latest work on the subject.

On p. 355 he says, "Um in diesen bedeutungsvollen Ergebnissen das sicher Festgestellte und das Hypothetische von einander zu halten, haben wir vorgeschlagen das Alexin oder Komplement unter dem Namen Cytase (d. h. zellenlösendes Enzym), die sensibilisierende Substanz dagegen unter dem Namen Fixator zu bezeichnen." He also states (p. 357) that Savtchenko was the first to show that red blood cells which are laden with *the specific fixateur* are extraordinarily easily phagocytosed.

* *Loc. cit.*, p. 111.

Savtchenko stated further that he took as the objects of experiment the phagocytes of the guinea-pig and its red blood corpuscles, and the serum of a rabbit immunised against the red blood cells of the guinea-pig, and heated the serum of the rabbit to 55° C. to destroy the alexines, leaving *the specific fixateur* intact. He also stated that he took the washed red cells of a guinea-pig and diluted them with normal saline solution and added a quantity of heated hæmolytic immune serum in a dilution of 1/200. After this mixture had been six hours at 37° C. he centrifugalised and washed the corpuscles thrice with normal saline. "The red blood cells," he adds, "had attached to themselves *the fixateur*; since the addition of normal serum was sufficient to bring about the solution of the hæmoglobin."

Again,* he states, "Il est possible qu'il existe dans le plasma un minimum de fixateur insuffisant pour être decélé par la réaction de dissolution, mais tout à fait suffisant pour provoquer la phagocytose après s'être fixé sur ces derniers."

Savtchenko's position is this: As the result of his experiments he came to the conclusion that in the serum of a rabbit immunised with guinea-pig's blood, there exists a substance which causes phagocytosis of the red blood cells of the guinea-pig, and this substance, which may act either on the phagocytes or on the bodies to be phagocytosed, is the specific fixateur, and possibly, according to the amount present in the serum, this substance causes hæmolysis or phagocytosis.

From what has been given here of Savtchenko's work, it appears to be beyond doubt that he considered that the specific fixateur which induces the phagocytosis of red blood cells is the same as the hæmolytic amboceptor of Ehrlich and not a separate body inducing this action.

Barratt† has shown that even with *unheated* immune serum, phagocytosis of red blood cells may occur without the serum possessing either hæmolytic or agglutinative properties, and concludes from this that the phagocytosis is not induced by the fixateur in the sense of the term as used by Savtchenko, nor by the agglutinin, but by some other body acting on the red blood corpuscles and not on the leucocytes. This body he placed in the class of "opsonins."

Besredka (13) in summing up Barratt's paper says, "Il y a, en effet, dans un sérum hæmolytique plusieurs substances. Est-ce le fixateur (amboceptor), qui détermine la phagocytose? est-ce l'agglutinine? est-ce enfin une troisième substance qui aurait uniquement pour fonction de présider à la phagocytose"? Besredka, it is clear, also assumes that the fixateur is identical with the amboceptor.

* *Loc. cit.*, p. 118. † *Loc. cit.*

The main question at issue, then, is whether the amboceptor, and by this I mean that acting in hæmolysis, is identical with the substance inducing the phagocytosis of erythrocytes—the opsonin of Wright and Douglas.

As Savtchenko and Barratt did not use exact quantitative methods in their experiments, and as such are desirable, it has been necessary to use a somewhat different technical procedure from that employed by these researchers, but the type of experiment was essentially the same as theirs.

Production of the Immune Serum, etc.

The materials used were the red blood cells of the ox, the serum of a rabbit immunised against these, and normal human leucocytes as the phagocytic agents.

The rabbit received intra-peritoneally doses of 10 c.c. of washed ox corpuscles at intervals of a week, 30 c.c. in all being administered before experiments were commenced.

The last injection was made on November 13, 1905. On the 27th of the same month it was found, testing in the usual way, that 0·002 c.c. of the serum produced, when fully complemented, total hæmolysis of 2 c.c. of a 5-per-cent. suspension in normal saline of washed ox corpuscles, after two hours at 37° C. and 12 hours at room temperature.

On the Effects of Heat on the Substances in the Serum which Induces Hæmolysis and Phagocytosis.

The first point to be studied was the influence of heat on the phagocytic action of the serum. With the *undiluted unheated* serum it was found to be a matter of considerable difficulty to perform phagocytic tests owing to hæmolysis somewhat obscuring phagocytosis. With the *undiluted unheated serum* only blood shadows were to be seen in the phagocytes, but on diluting the serum sufficiently to suppress the effects of the complement, hæmolysis was abolished and the red cells could be observed to be phagocytosed, apparently in their normal condition.

In order to find approximately at what degree of dilution hæmolysis would cease to come into play, a series of hæmolytic tests were performed in capillary pipettes. This method was employed in preference to that ordinarily adopted, because with Wright's method of performing phagocytic tests, to deal with absolute quantities is a matter of considerable difficulty.

Experiment.—Various dilutions of the *unheated* immune serum were made and equal parts of these dilutions and of a 5-per-cent. suspension of the washed red blood cells of the ox were mixed in a series of capillary pipettes, so that the ultimate proportion of serum in the mixtures varied from 1 in 2

to 1 in 100. These mixtures were placed at 37° C. for two hours. A parallel series was made with serum which had been heated to 55° C. for 15 minutes. This was placed in the same conditions as the former series.

Dilutions of serum in mixtures.	Result.
1— 2	Complete hæmolysis.
1— 6	Complete hæmolysis.
1— 10	Marked hæmolysis.
1— 20	Definite hæmolysis.
1— 30	Trace of hæmolysis.
1— 50	Trace of hæmolysis.
1— 60	Hæmolysis absent.
1— 70	Hæmolysis absent.
1—100	Hæmolysis absent.

This experiment shows that in the case of the *unheated* serum no hæmolysis took place in dilutions above 1 in 50 owing to dilution of the native complement and to the fact that no fresh complement was added. With the *heated* serum there was no hæmolysis, even with equal parts of serum and of the suspension of corpuscles, although in such a dilution the *unheated* serum produced complete hæmolysis.

It was therefore decided to begin phagocytic tests with dilutions about 1 in 50 in the case of the unheated serum.

Experiment to Show that Heating the Serum to 55° *C. to* 60° *C. Causes a Diminution of Phagocytosis.*

Unheated immune serum was diluted with normal saline solution in the proportions of 1 in 15, 1 in 20, 1 in 30. Of each of these dilutions one part was mixed in a capillary pipette with one part of a 5-per-cent. suspension of washed ox corpuscles and one part of washed human leucocytes, the final dilutions being approximately 1 in 45, 1 in 60, 1 in 90. The tubes were then placed for 15 minutes at 37° C., films being then made and stained with Leishman's stain.

At the same time series were made with portions of the serum which had been heated to 55° C. and 59° C. respectively. The final dilutions in these were 1 in 3, 1 in 6, 1 in 12, 1 in 24, 1 in 45, 1 in 60. A control consisted of one part of 0·85 saline, one part of the suspension of washed ox corpuscles and one part of washed human leucocytes.

It was found in the first few dilutions that so many red blood cells were taken up by the polymorphonuclear leucocytes, that the individual erythrocytes could not be distinguished and therefore the *percentage* of

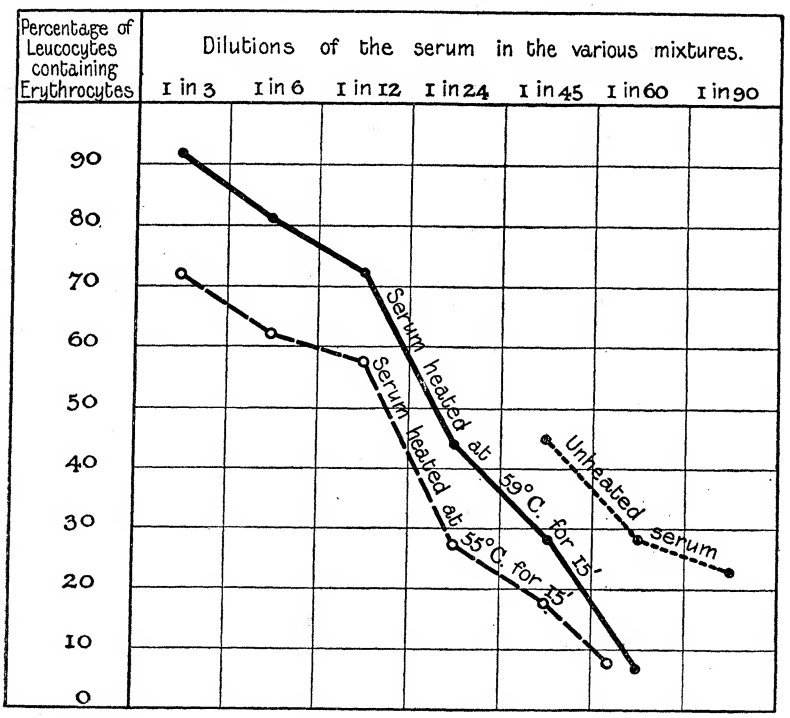
polymorphonuclear leucocytes containing red blood cells was taken as a criterion of the phagocytic action, 100 leucocytes being counted in each case. Some of the large mononuclear leucocytes contained occasionally one or two red cells, but these were so few as to be insignificant.

It was found that at corresponding dilutions the *unheated* serum produced a considerably greater amount of phagocytosis than did the *heated* and further that it bore greater dilution before giving up this property.

Using the above-mentioned method of enumeration the following results were obtained in the experiment.

Dilution.	Unheated serum.	Heated at 55° C.	Heated at 59° C.
	Per cent.	Per cent.	Per cent.
1 in 3	—	71	91
1 in 6	—	61	81
1 in 12	—	57	72
1 in 24	—	27	43
1 in 45	45	18	28
1 in 60	28	8	7
1 in 90	23	—	—

Saline control = 7 per cent. (The percentages refer to the number of polymorphonuclear leucocytes containing red blood cells.)



These results, which were confirmed by those of subsequent experiments, show that in an immune hæmolytic serum the substance inducing phagocytosis of the appropriate red blood cells is partially destroyed by heating the serum at 55° C. to 60° C. At the time of the experiment 0·002 c.c. of the serum when fully complemented produced complete hæmolysis of 2 c.c. of a 5-per-cent. suspension of washed ox corpuscles after two hours at 37° C. and 12 hours at room temperature.

Behaviour of the Hæmolytic Amboceptor towards Heat.

The next point for investigation was the influence of temperatures similar to those employed in the phagocytic tests, on the hæmolytic amboceptor.

Experiment.—Of the immune rabbit's serum two portions were taken, one being left unheated, the other being heated at 55° C. for 15 minutes. Into two series of test-tubes quantities of the serum ranging from 0·01 to 0·0001 c.c. were measured. One series then consisted of heated, the other of unheated serum. All the tubes were equalised in bulk by the addition of 0·85-per-cent. saline solution. To each tube 2 c.c. of a 5-per-cent. suspension of washed ox corpuscles were added with 0·2 c.c. of fresh normal guinea-pig's serum. One control consisted of 2 c.c. of the suspension of red cells with 0·2 c.c. of guinea-pig serum, and another of 2 c.c. of the suspension alone. The tubes were placed at 37° C. for two hours and were subsequently allowed to remain 24 hours at 0° C. The corresponding dilutions in the heated and unheated series showed the same degree of hæmolysis.

Result.

Unheated Serum.—Total hæmolysis with all quantities down to 0·005 c.c. Partial hæmolysis with all quantities down to 0·0001 c.c.

Heated Serum.—Total hæmolysis with all quantities down to 0·005 c.c. Partial hæmolysis with all quantities down to 0·0001 c.c.

In order to demonstrate conclusively whether there was any appreciable difference between the two series, von Fleischl's hæmometer was employed, the last three corresponding tubes in each series being compared with each other and with the guinea-pig serum control. The tubes were thoroughly shaken up and centrifugalised. The supernatant fluid was then pipetted off, and, if necessary, diluted sufficiently to give a reading between 20 and 60 on the scale before being placed in the chamber of the instrument. The reading found was then multiplied by the amount of the dilution.

The following are the results :—

	Colour index.
Guinea-pig serum control	64
Unheated serum—	
0·001 c.c.....	440
0·0005 „	155
0·0001 „	120
Serum heated at 55° C. for 15 mins.—	
0·001 c.c.	450
0·0005 „	220
0·0001 „	115

It is evident from these numbers that there is practically no difference between the colour indices of the two series; which permits the conclusion to be drawn that the hæmolytic amboceptor is *not* quantitatively diminished when the serum is heated at 55° C. for 15 minutes. Repeated experiments gave exactly similar results and it was found to be a matter of indifference whether the serum was heated *en masse* or in dilution, even in separate small quantities.

This fact is illustrated by the following experiment, which was performed to illustrate at the same time another point, namely, that there may be a large amount of hæmolytic amboceptor present in a *diluted* serum without the coexistence of the body inducing phagocytosis.

Experiment.—Four series of tests, A, B, C, D, each consisting of four tubes, were performed. Into successive tubes of each series 0·01, 0·005, 0·003, 0·002 c.c. of the immune serum was placed. The amounts were equalised by 0·85 saline solution. Series A and C were unheated. Series B and D were heated at 55° C. for 15 minutes. To each tube was then added 2 c.c. of a 5-per-cent. suspension of washed ox corpuscles, and to each tube of series A and B 0·2 c.c. of fresh guinea-pig serum (*i.e.*, one unheated, and one heated series was complemented). All the tubes were placed at 37° C. for two hours. It was then found that series A and B showed exactly corresponding degrees of hæmolysis.

Cubic centimetres.	A. Unheated and complemented.	B. Heated and complemented.
0·001	Almost complete	Almost complete
0·005	Marked	Marked
0·003	Slight	Slight
0·002	Slight	Slight

This first part of the experiment corroborates the result of the experiment mentioned immediately above.

In series C and D (the proportions of the serum in the mixtures corresponding to 1 in 220, 1 in 450, 1 in 730 and 1 in 1100 approximately), one series being heated and the other unheated, and both being uncomplemented, it was found that there was no sign of hæmolysis when these were compared with the controls, which were the same as in the previous experiment. The tubes of these two series were thoroughly shaken and centrifugalised. The supernatant fluid was pipetted off and the deposits washed thrice with 0·85 saline solution. To each deposit an equal quantity of normal saline was added. They were then well shaken and drawn up and down rapidly in capillary pipettes in order to produce a uniform suspension. Equal parts of each deposit and washed human leucocytes were mixed in capillary pipettes and placed 15 minutes at 37° C., films being then made and stained in the usual manner.

Result.—In no case was any phagocytosis observed, although in dilutions of 1 in 10 similarly treated, 93 per cent. of the polymorphonuclear leucocytes contained erythrocytes, which shows that such deposits can be phagocytosed, provided that the substance which induces phagocytosis is present in sufficient amount. Although in series C and D no phagocytosis occurred, yet in dilutions of 1 in 220 hæmolysis was almost complete in the complemented series, which shows that there must have been a large amount of hæmolytic amboceptor present, and that notwithstanding this large amount of amboceptor and an exposure during two hours of the red blood cells to it, no phagocytosis was observed.

This second part of the experiment then shows that in an immune *diluted* hæmolytic serum a considerable amount of hæmolytic amboceptor may be present without rendering the red cells capable of being phagocytosed.

This is supported by observations on non-immune hæmolytic sera. In the case of a guinea-pig's serum which was found in dilutions of 1 in 6 to produce slight hæmolysis of 2 c.c. of a 5-per-cent. suspension of the washed blood corpuscles of a rabbit, it was observed, that in phagocytic tests performed with the unheated serum, the human leucocytes used as the phagocytic agents contained in many cases blood shadows. These were found in 40 to 50 per cent. of the leucocytes in tests performed in the manner described in the former part of this paper. When, however, heated serum is employed no blood shadows are to be seen in the leucocytes nor is there any sign of phagocytosis.

In the case of the serum of an eel it was found that 0·01 c.c. produced after two hours at 37° C. marked hæmolysis of 2 c.c. of a 10-per-cent.

suspension of washed guinea-pig red cells. When heated at 55° C., however, such a serum failed to induce phagocytosis of the red cells after 15 minutes at 37° C., equal parts of the serum, of the suspension of red cells and of washed human leucocytes being employed.

All these facts then tend to show that the hæmolytic amboceptor may be present in a very considerable amount in a serum without giving to the latter the power of inducing phagocytosis of the appropriate red blood cells.

Conclusions.—The conclusion naturally come to is that the phagocytosis of red blood cells does not depend on the presence of the hæmolytic amboceptor, since:—

1. The substance which induces phagocytosis is partially destroyed by heat, while the hæmolytic amboceptor is entirely thermostable.

2. The hæmolytic amboceptor may be present in considerable amount in a hæmolytic serum without inducing phagocytosis, notwithstanding prolonged contact of the amboceptor with the red blood cells. This is contrary to the opinion of Savtchenko.*

Dean† has suggested that phagocytosis may be caused by a complement acting through an amboceptor, and that the partial destruction, of the property in the serum inducing phagocytosis, by heat may be due to the destruction of the complement, while the amboceptor, even in the absence of the complement, may still be capable of inducing phagocytosis. This theory, while it is difficult to disprove directly owing to the complement being destroyed at the same temperature as the thermolabile part of the substance inducing phagocytosis, seems to be an improbable one for the following reasons:—

(1) That it is not an action analogous to that of other amboceptors, *e.g.*, that concerned in hæmolysis. If one destroy the complement of a hæmolytic serum by heat, no hæmolysis takes place, notwithstanding the presence of the amboceptor in large amount.

(2) As has been shown above, the hæmolytic amboceptor may be present in large amount in a diluted serum, without that serum having the power of inducing phagocytosis even when Dean's method of testing is employed.

(3) In the dilution experiments recorded above it was shown that one may dilute the complement to such an extent as to abolish hæmolysis, and yet such a serum has a greater "opsonic" power in these dilutions than has the same serum when heated and employed in corresponding dilutions.

If the amboceptor act in the way Dean suggests, it must be supposed to

* *Loc. cit.*, p. 118.

† *Loc. cit.*

possess, in addition to its complementophilic group, another group which possesses the special function of inducing phagocytosis, *i.e.*, the amboceptor would combine the functions of the second and third receptor types of Ehrlich.

The experiments given in this paper, along with those of Barratt,* tend to show, that contrary to the opinion of Dean, Savtchenko was not correct in his conclusion that the specific fixateur, *i.e.*, the hæmolytic amboceptor, induced the phagocytosis of red blood cells, but that on the other hand it is much more probable that this phenomenon is caused by some special body belonging to the class of opsonins.

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REFERENCES.

1. Lohlein, "Sur la phagocytose 'in vitro' de microbes pathogènes," 'Annales de l'Inst. Pasteur,' 1905, vol. 19, p. 647.
2. Leishman, "Some experiments with 'stimulins,'" 'Trans. of Pathological Soc.,' 1905, vol. 66, p. 344.
3. Besredka, "Le sérum antistreptococcique et son mode d'action," 'Annales de l'Inst. Pasteur,' 1904, vol. 18, p. 363.
4. Wright and Douglas, "An experimental investigation of the *rôle* of the blood fluids in connection with phagocytosis," 'Roy. Soc. Proc.,' 1903, vol. 72.
5. Wright and Douglas, "Further observations on the *rôle* of the blood fluids in connection with phagocytosis," 'Roy. Soc. Proc.,' 1904, vol. 73.
6. Bulloch and Atkin, "Experiments on the nature of the opsonic action of the blood serum," 'Roy. Soc. Proc.,' 1905, vol. 74, p. 379.
7. Hektoen and Ruediger, "Studies in phagocytosis," 'Journ. of Infectious Diseases,' 1905, vol. 2, p. 128.
8. Dean, "An experimental enquiry into the nature of the substance in serum which influences phagocytosis," 'Roy. Soc. Proc.,' 1905, vol. 76, Series B, p. 506.
9. Wright and Reid, "On spontaneous phagocytosis," 'Roy. Soc. Proc.,' 1905.
10. Savtchenko, "Du rôle des immunisines (fixateurs)," 'Annales de l'Inst. Pasteur,' 1902, vol. 16, p. 106.
11. Barratt, "The phagocytosis of red blood corpuscles," 'Roy. Soc. Proc.,' 1905, vol. 76, Series B, p. 524.
12. Metchnikoff, in 'Kolle-Wassermann. Handbuch der pathogenen Mikro-Organismen,' 1904, vol. 4, p. 332.
13. Besredka, 'Bulletin de l'Inst. Pasteur,' 1905, vol. 3, p. 1037.

* *Loc. cit.*